

# **EXHIBIT 5**

# Virus-triggered acquired immunodeficiency by cytotoxic T-cell-dependent destruction of antigen-presenting cells and lymph follicle structure

(virus infection/antigen-presenting cells)

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**ABSTRACT** Virus-induced acquired immune suppression in mice infected with lymphocytic choriomeningitis virus is shown here to be caused by the CD8<sup>+</sup>-T-cell-dependent elimination of macrophages/antigen-presenting cells. Surprisingly, this is associated with severe destruction of the follicular organization of lymphoid organs, indicating a crucial role for dendritic cells and marginal zone macrophages in maintaining follicular structure. Once established, this immunopathology cannot be readily reversed by the elimination of CD8<sup>+</sup> effector cells. Such a T-cell-mediated pathogenesis may play a pivotal role in acquired virus-induced immunosuppression and may represent one strategy by which virus escapes immune surveillance and establishes persistent infections in initially immunocompetent hosts.

The noncytopathic lymphocytic choriomeningitis virus (LCMV), the infection of mice with LCMV, and the resulting consequences for this host (1-3) in some ways resemble human immunodeficiency virus (HIV) and HIV-triggered AIDS (4, 5) in humans. LCMV is non- or poorly cytopathic and replicates in many host cells (1-3, 6); some LCMV isolates have tropism for lymphoid cells, particularly dendritic cells and macrophages (7-9); and LCMV may cause severe immune suppression in mice (8, 10-15) and may establish persistent infections (1-3, 16-18).

Infection of mice i.v. with 10<sup>5</sup>-10<sup>6</sup> plaque-forming units (pfu) of the LCMV isolates LCMV-WE or LCMV-DOCILE has been shown (15) to cause severe transient-to-long-lasting immune suppression with respect to antibody and to cytotoxic T-cell responses (15). This acquired immune suppression was not caused by LCMV directly since congenitally or neonatally infected carrier mice exhibited normal immune responsiveness (15, 19, 20). Involvement of anti-LCMV cytotoxic T cells has been implied by the following findings: Anti-LCMV cytotoxic T cells cause immune suppression in adoptive transfer experiments when injected into LCMV-infected nude mice (15). Furthermore, *in vivo* treatment with anti-CD8 antibodies before or during infection was found to abrogate suppression (15). Both LCMV-WE and -DOCILE were shown to infect macrophages and dendritic cells but not CD8<sup>+</sup> T cells or B cells during acute infection (7, 8, 21, 22). In addition LCMV may infect a small percentage of CD4<sup>+</sup> T cells; this is readily detectable in carriers but not in mice during the acute phase of the infection (23).

We show that relative and absolute changes in lymphocyte subsets cannot readily explain immune suppression in mice infected with LCMV; however, the specific cytotoxic-T-cell-dependent elimination of LCMV-infected follicular dendritic cells and marginal zone macrophages causing complete de-

struction of lymph follicles correlated well with immune suppression.

## MATERIAL AND METHODS

**Virus.** LCMV isolates LCMV-WE and LCMV-DOCILE were obtained from F. Lehmann-Grube (Heinrich-Pette-Institut, Hamburg, F.R.G.) and C. Pfau (Rensselaer Polytechnic Institute, Troy, NY), respectively. Virus stocks were grown from a triple-plaque-purified second passage standard by using the low multiplicity of infection of 0.01 for 48 h on either L929 cells (LCMV-WE) or MDCK cells (LCMV-DOCILE). Virus was quantified as described (24). Vesicular stomatitis virus (VSV) serotype New Jersey (VSV-NJ) (Pringle isolate) was obtained from D. Kolakofsky (University of Geneva) and was used as described (15). Vaccinia virus (Lancy isolate; Serum und Impfinstitut, Bern, Switzerland) was used as described in detail elsewhere (13).

**Mice.** Inbred C57BL/6 (H-2<sup>b</sup>) mice were purchased from the Institut für Versuchstierkunde, University of Zurich. Mice were kept and experiments were performed according to the rules and permissions for animal experimentation of the Kanton Zürich.

**Lymphocyte Analysis.** Analysis of lymphoid cells was performed on an EPICS IV (Coulter) as described (24). The following rat monoclonal antibodies (mAbs) were used as culture supernatants for flow cytometry analysis and for immune histological staining: anti-macrophage F4/80 mAb (25) and anti-CD4 YTS191 and anti-CD8 YTS169 mAbs (26). A fluorescein-labeled goat anti-mouse IgM was used and a second-stage goat anti-rat IgG was used whenever directly labeled antibody was not available.

**Antibody Treatment.** Mice were treated as indicated with 0.2 ml of a tested rat anti-CD8 mAb (27, 28) or with 0.2 ml of a pretested polyclonal sheep anti-tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) antiserum (able to neutralize 6  $\times$  10<sup>4</sup> units of TNF- $\alpha$  *in vitro*) as described (29, 30).

**Antigen Uptake by Macrophages.** Commercial indian ink was centrifuged at 3000 rpm in a 30-cm diameter swing-out rotor and used at a 1:10 dilution, 0.2 ml i.v. per animal. Mice were sacrificed 2 h after injection. VSV-NJ antigen was prepared from Vero cell monolayers infected with VSV-NJ at a multiplicity of infection of 5-10 for 24 h. Cells were harvested, washed, pelleted, and resuspended as a 2% (wt/vol) suspension in a balanced salt solution. This preparation was frozen and thawed three times and sonicated, and debris was centrifuged out at 3000  $\times$  g for 10 min. The supernatant

Abbreviations: LCMV, lymphocytic choriomeningitis virus; HIV, human immunodeficiency virus; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; pfu, plaque-forming unit(s); VSV, vesicular stomatitis virus; mAb, monoclonal antibody.

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was UV-irradiated for 5 min with a germicidal lamp to inactivate all replicating virus. This supernatant was stored frozen and 0.2 ml was injected i.v. 2 h before sacrifice of mice.

**Immunohistochemistry Procedures.** Frozen tissue sections 5  $\mu$ m thick were fixed on slides in acetone for 10 min. The following primary reagents were used: goat anti-mouse IgM antiserum, peroxidase-labeled (Tago; diluted 1:200); rat ascites fluid containing mAb F4/80 [anti-mouse macrophage, diluted 1:80 (25); a gift of S. Gordon, Oxford, U.K.]; two mAbs developed in this laboratory, rat anti-*LCMV* mAb VL-4 and rat anti-VSV-NJ mAb 17-2-A3.1 with a specific neutralizing titer of  $>1:10,000$ . The rat anti-marginal zone macrophage mAb MÖMA-1, diluted 1:30 (31), and the follicular-dendritic-cell-specific antibody 4C11, diluted 1:20 (32), were gifts of W. van Ewijk (University of Rotterdam) and G. Kraal (University of Amsterdam) and of M. A. Kosco and D. Grey (Basel Institute of Immunology), respectively. Primary antibodies were detected by an indirect immunoenzymatic staining procedure using peroxidase-labeled rabbit anti-goat IgG antiserum (DAKO, Denmark; diluted 1:40) or goat anti-rat immunoglobulin (Tago, diluted 1:30), followed by alkaline phosphatase-labeled donkey anti-goat immunoglobulin (The Jackson Laboratory; diluted 1:30). Sections were counterstained with Meyer's hemalum (Kantons-apotheke, Zurich) for 2 min. Formaldehyde-fixed and paraffin-embedded sections were stained with hematoxylin/eosin.

## RESULTS

**CD4<sup>+</sup> and CD8<sup>+</sup> T Cells and B Cells in LCMV-Infected Mice.** To evaluate whether lymphocyte subsets exhibited changes during the LCMV-induced immune suppression that parallel changes seen in AIDS patients, CD4<sup>+</sup> and CD8<sup>+</sup> T cells and B cells were monitored in mice and compared to congenital LCMV carriers or vaccinia virus-infected mice and uninfected controls (Fig. 1). Relative and absolute levels of T-cell subsets did not vary significantly in vaccinia virus-infected mice; however, considerable changes were observed in LCMV-infected mice. Splenic CD8<sup>+</sup> T cells rose from  $8 \times 10^6$  to  $5 \times 10^7$  cells on day 8 or day 12, whereas the absolute numbers of CD4<sup>+</sup> T and B cells changed less drastically,

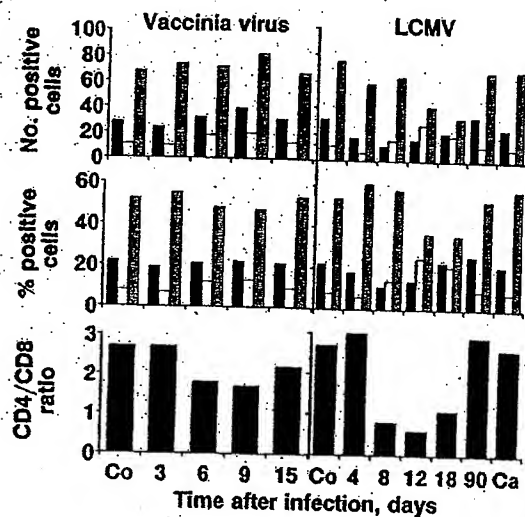


FIG. 1. Splenic lymphocyte profiles of acutely infected mice. Groups of three C57BL/6 mice were left uninfected (bars Co) or were infected at various times before sacrifice. Mean values are shown; SEM values were  $<5\%$ . Carrier mice (bars Ca) were LCMV-positive offsprings of neonatally infected C57BL/6 mice. (Top and Middle) Bars: solid, CD4<sup>+</sup>; open, CD8<sup>+</sup>; stippled, IgM. The number of positive cells ( $\times 10^6$ ) is shown.

confirming earlier analyses with lower-dose LCMV infections (33, 34). This resulted in an overall increase of the spleen size and an inversion of the CD4<sup>+</sup>/CD8<sup>+</sup> ratio on days 8–12. Analysis of the time course of lymphocyte subset ratios in blood and spleens of LCMV-infected mice up to 50 days after infection revealed that the CD4<sup>+</sup>/CD8<sup>+</sup> ratio was back to about normal on day 18 in blood and after day 28 in spleens. Thus despite a low CD4<sup>+</sup>/CD8<sup>+</sup> ratio and decreased relative CD4<sup>+</sup> T-cell frequencies, absolute numbers of CD4<sup>+</sup> T cells or B cells were within 30–50% of normal ranges; the low CD4<sup>+</sup>/CD8<sup>+</sup> ratio could, therefore, not readily explain the severe immune suppression observed in LCMV-infected mice.

**Histological Analysis of Peripheral Lymphoid Tissue in LCMV-Infected Mice.** Evaluation of spleen and lymph node sections by conventional and immune histology revealed extensive destruction of lymph follicle structures in LCMV-infected euthymic mice (Fig. 2). The normal orderly arrangements of F4/80-positive macrophages and of IgM-positive B cells in lymph follicles of uninfected mice (Fig. 2a) were also seen in spleens (and lymph nodes, data not shown) of LCMV carrier mice (Fig. 2f) and uninfected (Fig. 2g) or LCMV-WE-infected (Fig. 2h) nude mice. The latter findings confirm that LCMV alone has no adverse effects on the integrity of lymphoid tissue (7). In addition spleens obtained from adult euthymic mice infected 4 days before analysis (Fig. 2b) exhibited normal follicular structure; however, by day 6 (Fig. 2c) and, more pronounced, by day 8 (Fig. 2d), the follicles had disintegrated and had largely disappeared morphologically. Follicle-like structures reappeared slowly and variably after days 14–20 of infection (Fig. 2e). As shown earlier (15), measurable antibody-dependent immune responses to third-party antigens were absent from day 7 to day 14 of LCMV infection; they were gradually reinstated in parallel with the histological recovery of follicular structures in spleens (and lymph nodes; data not shown). It is noteworthy that viral antigen, as revealed by appropriate staining, peaked around days 4–8 in parallel with viral titers measured by plaque formation (day 6,  $10^8$ – $10^9$  pfu/g of spleen; day 14,  $10^2$ – $10^4$  pfu/g of spleen). In mice treated with a rat anti-mouse CD8 mAb either before or shortly after infection with LCMV-WE, the decay of follicular structure did not occur or was drastically reduced (Fig. 2i and n, upper micrographs) when compared to LCMV-infected and not anti-CD8-treated animals (Fig. 2i, k, and n, lower micrograph). Correspondingly, neutralizing antibody responses against VSV were within normal ranges (i.e., IgG titers of 1:1280–1:5120 on day 8) in LCMV-infected mice treated with anti-CD8 before or during initiation of infection (ref. 15 and data not shown). If treatment started later than day 6 or 7 after infection, the destruction of lymphatic architecture was not inhibited (data not shown, but comparable to Fig. 2i and k) and immune suppression of antibody responses was not prevented (i.e., IgG titers of  $<1:80$  on day 8). Treatment on days 4, 6, 8, and 10 with a potent sheep anti-TNF antiserum that has dramatic effect on resistance to *Listeria* (30) did not prevent LCMV-induced suppression of IgG antibody responses to VSV (i.e., IgG titers of  $<1:80$  on day 8). These results are compatible with earlier studies indicating that TNF was not instrumental in causing lymphocytic choriomeningitis (29) and suggest that TNF may not be crucially involved in mediating this acquired immunodeficiency.

**Histological and Functional Analysis of Macrophages and Dendritic Cells.** Since LCMV is expressed in  $<0.1$  to 1% of peripheral CD4<sup>+</sup> T cells (22, 23, 35) in acutely infected mice, macrophages and dendritic cells are the most likely targets for immunopathological destruction since they represent the bulk of the infected cells in spleen and lymph nodes (6, 7) (Fig. 3c and d). This was illustrated by reduced staining with macrophage-specific antibodies (Figs. 2d and 3f and g) and

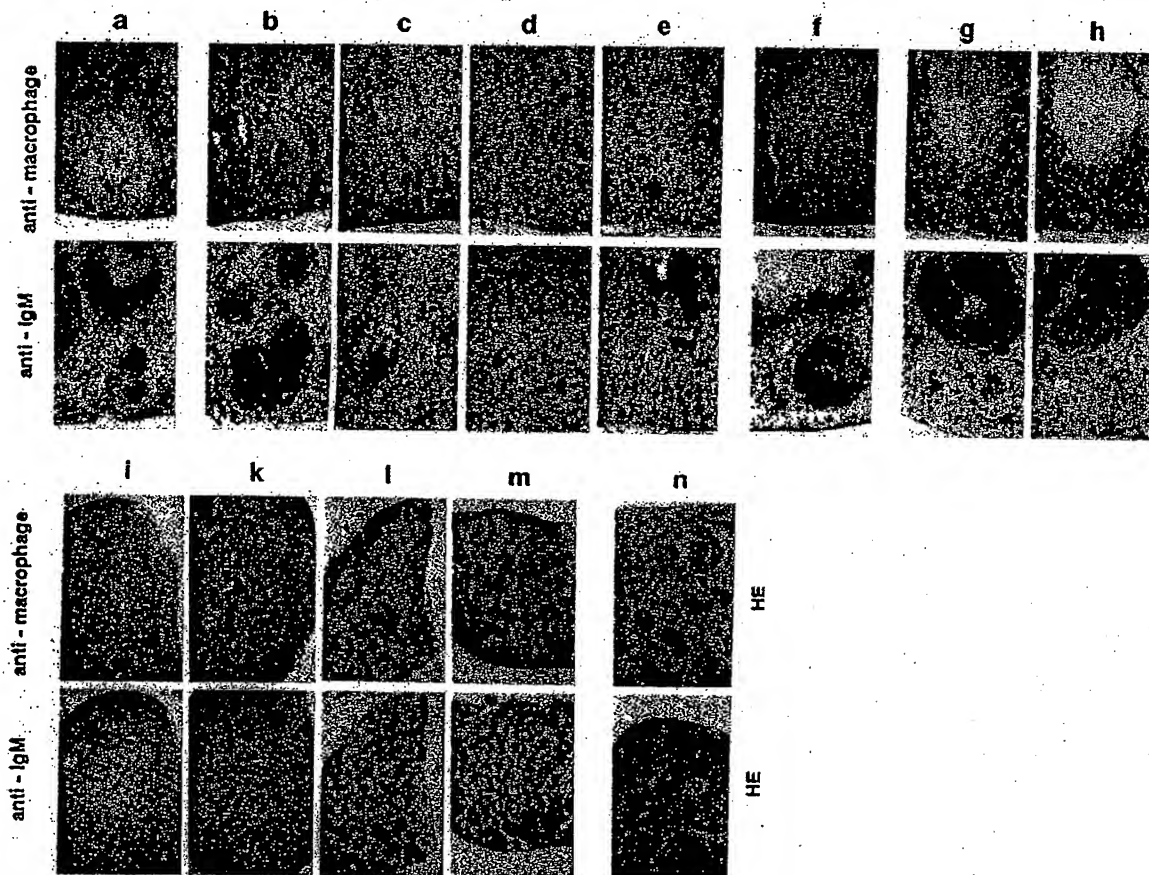


FIG. 2. (a-h) Immune-histological sections of spleens from mice infected with LCMV-WE for various time periods: (a) Uninfected control C57BL/6. (b-e) LCMV-WE-infected mice [ $1 \times 10^6$  pfu, i.v. 4 days (b), 6 days (c), 8 days (d), or 14 days (e) earlier]. (f) Congenital LCMV carrier. (g) Uninfected *nu/nu* C57BL/6. (h) LCMV 8-day-infected *nu/nu* mouse. Frozen sections were stained with anti-macrophage mAb F4/80 (upper micrographs) and anti-IgM (lower micrographs) and counterstained with hemalum. C57BL/6 mice were infected with  $5 \times 10^3$  pfu of LCMV-DOCILE (i-l and n) (note that i and k are sections from two independent animals treated identically) or were not infected (m). Staining of frozen sections was with anti-macrophage mAb F4/80 (upper micrographs), with anti-IgM (lower micrographs), or with hematoxylin/eosin (HE) on conventional histological sections (n). Mice had been treated with control antibody [i, k, and n (lower micrograph)] or with anti-CD8 antibody [l, m, and n (upper micrograph)] and were sacrificed on day 9 after infection. (a-h,  $\times 180$ ; i-n,  $\times 20$ .)

by functional studies (Fig. 3 a, b, and e). Spleens of mice 9 or more days after infection with LCMV showed little uptake of carbon (Fig. 3a, lower micrograph) or of inactivated VSV antigen, particularly by marginal zone macrophages (Fig. 3b, lower micrograph) when compared to uninfected control mice (Fig. 3 a and b, upper micrograph). When LCMV-infected mice were treated early with anti-CD8, carbon uptake was within normal ranges (Fig. 3e, upper micrograph). This correlated well with the LCMV-WE infection of marginal zone macrophages and follicular dendritic cells in germinal centers of mice treated with anti-CD8 before or early in LCMV infection (Fig. 3 c and d, upper micrographs), whereas no or very few such infected cells were found in mock-treated infected mice (Fig. 3 c and d, lower micrographs). Some apoptotic cells and pyknotic nuclei were seen on days 6–10 in splenic macrophages of LCMV-infected mice signaling cell destruction (Fig. 3e, lower micrograph). Staining with specific antibodies revealed that marginal zone (so called metallophilic) macrophages and follicular dendritic cells were virtually absent on day 9 of an immune-suppressive LCMV infection (Fig. 3 f and g, lower micrographs) when compared to uninfected controls (Fig. 3 f and g, upper micrographs) or CD8-depleted uninfected (Fig. 3h, upper micrograph) or LCMV-infected mice (Fig. 3h, lower micrograph). In contrast, red pulp macrophages were less organized and dispersed but present in substantial numbers (Fig. 2 c, d, and e, upper micrographs; data not shown).

## DISCUSSION

The present evidence suggests that LCMV-induced suppression of antibody responses (8, 10, 11, 15, 36) may be caused by the T-cell-dependent destruction of macrophages, particularly in the marginal zone, and of follicular dendritic cells, which are crucially involved in mounting immune responses (37–39). A key finding of the present study is that these cells are apparently instrumental in maintaining the typical follicular organization in spleens and lymph nodes. Although evidence that antiviral  $CD8^+$  T cells act cytolytically by cell contact *in vivo* is indirect (40–42), such an effector mechanism could explain the findings adequately. However, it cannot be excluded formally yet that T-cell-dependent secreted factors [such as  $TNF-\alpha$ , interleukin 10, transforming growth factor  $\beta$ , etc. (43–45)] may be involved in destruction of macrophages and antigen-presenting cells. Since treatment with an anti- $TNF-\alpha$  antiserum could not inhibit suppression,  $TNF-\alpha$  alone is not sufficient. Ongoing adoptive transfer experiments indicate that lymphocytes from LCMV-suppressed mice function well *in vitro* or *in vivo* alone or mixed with normal lymphocytes in irradiated normal recipients. These studies further support the hypothesis that suppression is not caused by factors or active suppression and are compatible with the view that antigen presentation and, therefore, induction and triggering of T cells may be defective in acutely LCMV-infected normal mice. A possible  $CD8^+$ -T-cell-dependent pathogenesis of AIDS has been pro-

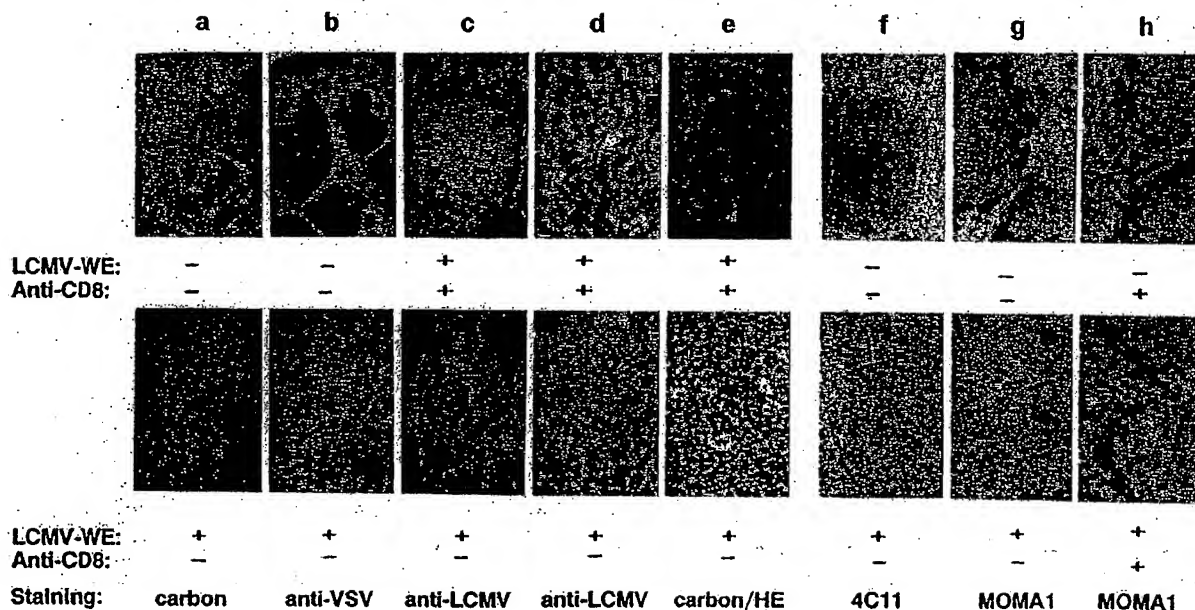


FIG. 3. Prevention of immunopathological destruction of infected macrophages and dendritic cells and preservation of antigen uptake and of follicular structure by anti-CD8 treatment. C57BL/6 mice uninfected or infected with  $2 \times 10^6$  pfu of LCMV-WE were treated as shown with control antibody or anti-CD8 antibody on days 0, 2, and 4 and sacrificed on day 9 after infection. At 2–3 h before sacrifice either 0.2 ml of 1:100 diluted indian ink [a and e (upper micrograph)] or inactivated VSV antigen (b) was injected. Staining of frozen sections was with hematoxylin only (carbon) [a and e (upper micrograph)], with a rat anti-VSV antibody (b), or with anti-LCMV (c and d) antibody; a conventional histological section was stained with hematoxylin/eosin [e (lower micrograph)]. Follicular dendritic cells were stained with mAb 4C11 (32) (f) and marginal zone (metallophilic) macrophages were stained with mAb MOMA-1 (31) (g and h) in spleens of uninfected controls [f and g (upper micrographs)], of LCMV-WE-infected C57BL/6 on day -9 [f–h (lower micrographs)], or mice that had been treated on days 0, 2, and 4 with anti-CD8 antibody (h). +, Added; –, not added. [a and b,  $\times 85$ ; c and e (upper micrograph) and f–h,  $\times 180$ ; d and e (lower micrograph),  $\times 290$ .]

posed to explain reduction of infected or HIV-antigen-binding CD4<sup>+</sup> T cells (46, 47). It is conceivable that in analogy to the immunopathology observed during a LCMV infection, virus-specific cytotoxic T cells (and probably not the virus itself) may be responsible for both numerical and functional reduction of macrophages and antigen-presenting cells and thus may cause destruction of follicular structures in HIV infections (48–53). Detailed histopathological studies may be taken to support the hypothesis that CD8<sup>+</sup> T-cell-dependent immunopathology may significantly contribute to the pathogenesis of AIDS; lymph node histopathology in patients with AIDS-related complex is often strikingly similar to that of mice suffering from LCMV-induced immunosuppression as shown here. CD8<sup>+</sup> T-cell immunity is apparently instrumental in the control of HIV spread (4, 46, 47). Direct evaluation of the involvement of CD8<sup>+</sup> T cells in HIV-infected individuals and in AIDS pathogenesis is not feasible because it might interfere with immunity to possible superinfections and, therefore, be potentially harmful for the patient. Additionally, as the data obtained in the mouse model suggest, depletion of CD8<sup>+</sup> T cells would not have an immediate beneficial effect on immunosuppression. However, there may be gradual recovery over an extended period of time (see Figs. 1 and 2a), which at least in the mouse was dependent on both host and virus strain.

Why should LCMV, HIV, or other viruses be immunosuppressive? The CD8-dependent severe immune suppression caused by some LCMV isolates is accompanied not only by a severe suppression of antibody and of cytotoxic-T-cell responses to other viruses but also by a drastic reduction of anti-LCMV T-cell responses themselves, measurable after day 7 or 8 in LCMV-infected mice (1–3, 54–56). In fact, both the extent of immunopathology and of immunosuppression have so far correlated with the known characteristics of various LCMV isolates. Neurotropic LCMV strains that do not cause suppression of their own cytotoxic T-lymphocyte responses show little histological damage, whereas the vis-

cero-lymphotropic isolates induce both high levels of suppression and immunopathology. The experiments presented suggest that T-cell-dependent immunopathology of lymphoid tissue correlated well with immunosuppression and, therefore, may contribute to it; this process may be enhanced and maintained by selection of appropriate virus variants exhibiting antigen or T-cell epitope changes or varying tropisms for lymphoid or nonlymphoid cells (24, 57, 58). Collectively these mechanisms may permit virus to persist in initially immunocompetent hosts.

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